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Controlling Ribozyme Activity by Metal Ions

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Running title: Metals Controlling Ribozyme Activity

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Abstract

The observed rates of ribozyme cleavage reactions are strongly dependent on the nature of the metal ion present. Metal ions can thereby exhibit a strong inhibiting or accelerating effect compared to Mg^{2+}, which is usually considered the natural cofactor. Alkaline, alkaline earth, transition, d^{10}, and other metal ions are applied either to gain a spectroscopic handle on the metal center, and/or to elucidate the catalytic mechanism. Here we shortly review some of the most recent publications on the influence of different metal ions on catalysis of the hammerhead, hepatitis delta virus and group II intron ribozymes. Comparison of the observed cleavage rates of hammerhead ribozymes with the metal ion affinities of different ligands reveals that these rates correlate perfectly with the intrinsic phosphate affinities of the metal ions involved.

Introduction

RNA or DNA in a living cell is always associated with metal ions (M^{n+}) [1]. The intrinsic negative charge of the phosphate sugar backbone needs to be compensated not only to achieve regular duplex formation but even more for complex three-dimensional architectures. Estimates show that a 200 nt long RNA needs to overcome repulsive forces of about 2'100 $kJmol^{-1}$ to fold in the absence of metal ions [2]. The vast majority of M^{n+} are diffusely bound to RNA, i.e. via unspecific interactions that can best be described by a non-linear Poisson-Boltzman distribution [3,4]. An estimated 10% of the negative charge are compensated by specifically bound M^{n+}, as has been concluded from X-ray structures of larger RNAs [5]. In RNA, a distinct network of inner-sphere (i.e. direct) and outer-sphere (i.e. mediated through water molecules) coordination is built to hold metal ions in place. A M^{n+} binding pocket thereby offers an excess of possible coordination and H-donor/acceptor sites (Figure 1). Outer-sphere binding should thereby not be equated with unspecific binding: If part of a tight hydrogen bonding network, the coordinated water molecules (and thus also the central metal ion) are strongly bound, as is very well known from the spine of hydration of DNA [6].

K^+ and Mg^{2+} are most abundant in living cells and thus believed to be the metal ion-cofactors associated with nucleic acids. Originally used as mimics for Mg^{2+}, other divalent metal ions have been applied in uncountable studies to, e.g. elucidate the catalytic mechanism or to get a spectroscopic handle on the metal ion. Thio-rescue [7,8], related NAIM/NAIS experiments (nucleotide analogue interference mapping/suppression) [7,9,10], and EPR measurements are just three examples [11-13]. Such metal ions include alkaline earth ions (Ca^{2+}, Sr^{2+}, Ba^{2+}), first row transition ions (Mn^{2+}, Co^{2+}, Ni^{2+}), group 12 (IIB) ions (Zn^{2+}, Cd^{2+}), main group metal ions (Pb^{2+}),

all lanthanide ions, as well as complexes like $Co(H_2O)_6^{3+}$. Changing the metal ion usually strongly affects ribozyme activity. Classically, an inhibiting effect is expected when Mg^{2+} is partly or fully replaced but also tremendous rate accelerations have been observed. These rate modifications allow to correlate activity with chemico-physical properties of the ions, e.g. ionic radius, pK_a of coordinated water, or general affinities, and thus help to elucidate the mechanism [14,15]. Unfortunately, only rarely clear correlations are found.

In this short review we focus on some of the most recent results on the influence of different metal ions on the cleavage rates of tertiary stabilized hammerhead (HH), hepatitis delta virus (HDV), and group II intron ribozymes. We discuss and compare these data with available results from the literature on smaller nucleic acid systems or derivatives. Interestingly, a clear correlation of HH ribozyme cleavage rates and the intrinsic affinities of the respective metal ions towards phosphate groups is revealed.

Replacing Mg^{2+} by monovalent ions

More than 25 years ago, a two-metal ion mechanism has been proposed for dephosphorylation reactions [16,17] and is now often considered as one of the favored modes of action for ribozymes [18,19]. With the discovery that small ribozymes are also active in the presence of monovalent metal ions only, e.g. alkaline ions but also NH_4^+, this dogma has been strongly opposed for some time. However, given the facts that (i) alkaline ions are also metal ions, (ii) non-physiological molar concentrations have to be applied for folding and activity, (iii) the catalytic activity in the presence of M^+ ions could in no case rival the one observed with M^{2+} ions, and (iv) the realization that metal ions need not to be coordinated directly but can exert their crucial influence also from a distance [1], it becomes again increasingly evident, that ribozymes simply are obligate metallo-ribozymes.

It is well known that high Na^+ concentrations can replace Mg^{2+} or Zn^{2+} in the related hydrolysis of ATP [20,21]. 500 times higher concentrations of M^+ ions are needed in this simple system to compensate for the intrinsic much lower affinity of M^+ ions compared to M^{2+} ions. In larger ribozymes, a larger excess of M^+ is needed. The replacement of one Mg^{2+} ion by several Na^+ ions has recently been implicated by a molecular simulation study of the HH ribozyme, where a large negatively charged binding pocket has been located at the active site [22]. MD simulations in the absence of divalent ions revealed two Na^+ ions sitting in the catalytic pocket replacing one Mg^{2+} ion, but not occupying distinct coordination sites [23]. Consistently, the *Schistosoma* HH ribozyme can globally fold in the presence of M^+ ions, but M^{2+} ions are still needed for efficient catalysis [24]. Similar results were obtained for the HDV ribozymes in the presence of molar concentrations

of M^+ ions, Li^+ yielding 30-50 x faster cleavage rates than Na^+ and NH_4^+ [25]. However, additional presence of Mg^{2+} further accelerates cleavage by about 100 times [25]. Along the same line, crystallographic studies of the HDV ribozyme have demonstrated that the location of monovalent and divalent ions do not overlap completely [26]. All these results suggest that accumulated M^+ ions can partly substitute for Mg^{2+}, but they will not reach the equivalent electrostatic or polarizing potential either due to their lower charge density, their lower affinity, or both.

Replacing Mg^{2+} by Ca^{2+}

The HDV ribozyme exists in two forms, the genomic and the antigenomic, being encoded one on each of the two viral RNA strands. These two forms exhibit an interesting Mg^{2+}/Ca^{2+} switch. Whereas the genomic HDV ribozyme cleaves faster in the presence of Mg^{2+}, Ca^{2+} is the better cofactor for the antigenomic form [27]. The nature of the nucleotide 5' to the cleavage site (position –1) (U in the genomic and C in the antigenomic HDV ribozyme) is decisive for this specificity, as changing C→U or U→C, respectively, reverses the Mg^{2+}/Ca^{2+} preference [27]. The reason for this switch in M^{n+} preference is unknown, but perhaps due to the different hydrogen bonding properties of these two nucleobases the outer-sphere network of the two ions is altered.

The HH ribozyme is one of the best investigated catalytic RNAs. However, despite numerous crystal structures and uncountable biochemical studies, the mechanism of action is still far from being understood [19,28]. The HH ribozyme is active in the presence of transition metal ions as well as at high M^+ concentrations. Since the discovery of the much more active extended (or tertiary stabilized) hammerhead ribozyme [29,30], several studies dealt with the influence of metal ions on catalysis and folding. Out of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}, Mg^{2+} works best, the alkaline earth metal ions showing an inverse correlation between k_{obs} and the pK_a of the hydrated ions.

Group II introns rank among the largest catalytic RNAs and implicitly require Mg^{2+} for catalysis [31]. No group II intron is known where the addition of a divalent metal ion other than Mg^{2+} leads to an increase in activity. The yeast Sc.ai5γ group II intron and its derived ribozyme constructs are among the best investigated large ribozymes [32]. Ca^{2+} has been shown to be an efficient inhibitor of the first step of splicing of the Sc.ai5γ derived D135 ribozyme: In the presence of 5% Ca^{2+} (95% Mg^{2+}), the catalytic rate k_{cat} drops by about 50% and at 20% Ca^{2+} no activity is detectable anymore [33]. A replacement of Mg^{2+} by Ca^{2+} in the catalytic core can not be excluded to-date. However, recent single molecule Fluorescence Resonance Energy Transfer (smFRET) experiments have provided unprecedented insights into the influence of metal ions on folding of large RNAs [34,35]: (i) The global architecture of these RNAs is severely disturbed upon addition of Ca^{2+}. (ii) Ca^{2+}

induces the formation of a second subpopulation that behaves distinctly differently, both in folding pathway and smFRET states. The number of these type 2 molecules thereby increases linearly with Ca^{2+} concentration. Interestingly, every single molecule is forced into one subpopulation and cannot switch between them. By these experiments, for the first time a functional role for folding heterogeneity could be demonstrated [35]. The observation that not all molecules are evenly affected by Ca^{2+}, hints that Ca^{2+} coordinates cooperatively. As the total concentration of Ca^{2+} by far exceeds the one of the single molecules, this means that cooperativity only becomes effective after first binding events have taken place with Ca^{2+}.

Replacing Mg^{2+} with transition and d^{10} metal ions

In the HH ribozyme all transition metal ions investigated promote faster catalysis than Mg^{2+}, with Mn^{2+} promoting catalysis about 400 times more than the one reached by Mg^{2+} [15]. The reason for these large differences in reaction velocity is still unclear. Crystal structures of hammerhead ribozymes in the presence of Mg^{2+}, Mn^{2+} and Cd^{2+} revealed subtle differences in binding (Figure 2) [36,37]: All three ions occupy roughly the same regions but coordinate slightly differently. A close-up of the active site reveals small, but possibly decisive differences of inner-sphere (Mn^{2+}) versus outer-sphere coordination (Mg^{2+}). Along the same line, recent molecular dynamics simulations of five different hammerhead states have revealed a binding pocket with a high electronegative charge potential that attracts metal ions, but binds them differently in mode and number due to the large enough pocket and excess of possible liganding sites present (Figure 1) [22]. However, different mechanisms might take place depending on the metal ion present: For example, a single-proton transfer was suggested to occur in the presence of Mg^{2+} or Mn^{2+}, whereas a double-proton transfer might take place with Cd^{2+} in the case of the RzB HH ribozyme [15]. As an acid, either a solvated Mg^{2+} ion or a guanine-N1H, both with a perturbed pK_a value have been suggested. The origin of perturbed pK_as in complex RNAs is largely unknown. From nucleobase studies, coordinating M^{n+} are well known to shift pK_as into the physiological range as was recently reviewed (Figure 3) [38]. Indeed, in the VS ribozyme it was recently reported that metal ions control a nucleobase pK_a [39]. The polarizing effect of Mg^{2+} is expected to be similar to that of a kinetically stable M^{n+} like Pt^{2+}, if Mg^{2+} is held tightly in place by further coordination. The largest effect on pK_a is actually enforced by protons, e.g. guanine-N7 protonation shifts the pK_a of N1H to 7.22 (Figure 3) [40].

Recently, the effect of Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Na^{+}, and NH_4^{+} was investigated not only on catalysis but also on folding of the *Schistosoma mansonii* HH by FRET [24]. All M^{n+} are capable of globally folding the HH ribozyme, but 10'000 fold differences in cleavage rates are

observed, Mn^{2+} again being the best cofactor [24]. When folded in 2M Na^{+}, still a >4600 fold activation of cleavage can be observed for Mn^{2+} compared to Mg^{2+}. Taken together, this is strong indication for a direct involvement of the metal ion in optimal cleavage activity.

The change in cleavage rate follows the phosphate affinity of the applied metal ion

Correlations of the observed catalytic rates k_{obs} with ionic radii, pK_a values of the hydrated metal ion complexes, the Irving-Williams series (Figure 4A) or others have been attempted in the past. No clear correlation was observed for all studied metal ions, again suggesting that different mechanisms might occur. In this regard, single and double proton transfer mechanisms involving either hydrated metal ions or ionized nucleobases are being considered [19,28,40,41]. Considering the intrinsic coordination chemical properties of the metal ions involved, it is actually very unlikely that any straight trends are observed: (i) Along the Irving-Williams series not only the affinity of metal ion-ligand interactions increases, but also the preference for oxygen ligands changes to a clear preference for nitrogen sites. Both types of liganding sites are abundant in metal ion binding pockets within nucleic acid structures (Figures 1 and 3). (ii) At the same time, the preference for inner-sphere coordination often increases from left to right along the same series of ions (which is not true for phosphate ligands). (iii) The Irving-Williams series is not strictly followed for phosphate ligands, which are the most important coordination sites for metal ions in nucleic acids (Figure 4) [42,43]. Most importantly, the phosphate affinity *decreases* from Mn^{2+} to Ni^{2+} (most likely due to outer-sphere binding).

Indeed, when plotting the k_{obs} values of RzB HH cleavage [15,24] versus the M^{n+} affinities towards a phosphate monoester, a surprisingly good correlation is observed (Figure 4B). The k_{obs} values observed with the alkaline earth M^{2+} and Mn^{2+} fit well to the Irving-Williams series as well as to the phosphate affinities. The additionally investigated transition and d^{10} M^{2+} do clearly not follow anymore the Irving-Williams trend but almost perfectly the one of the phosphate affinities with Mn^{2+} exhibiting both the highest k_{obs} and affinities (Figure 4). As the nucleotide affinities are strongly dominated by the phosphate-M^{n+} interaction [5,42], this result shows that the strength of the phosphate coordination dominates the effect of metal ions on catalysis in at least the HH ribozyme.

Conclusions

The nature of divalent metal ions obviously is the determining factor how RNAs fold and what mechanism is employed. How this is achieved is still lying mainly in the dark – especially

concerning the picture on the atomic level. The above summarized recent results on the effect of varying metal ions on ribozyme activity now trigger several considerations:

(i) There is increasing evidence that ribozymes use different mechanisms depending on the outside conditions, e.g. the kind and concentration of metal ions employed. As a consequence this means that the employment of mimics, e.g. Cd^{2+} instead of Mg^{2+}, might not reflect the "real" catalytic mechanism but rather a minor or very different pathway. Such concerns have increasingly been raised in the past years, e.g. for HH ribozymes [10]. The transfer of such results to the wild-type system should thus always be performed with care.

(ii) Complex RNA structures are surprisingly selective for a given metal ion, i.e. they recognize it with high specificity and selectivity. One could argue that such specific binding pockets exist just by chance or based on pure statistics. On the other hand Nature is very efficient and has had millions of years to optimize its processes. In evolutionary terms, one can imagine that there is hardly any chance that the highly effective binding of various metal ions and their considerable effects on ribozyme activity is without any purpose. This is even more so, as such effects are not restricted to only one specific ribozyme, but seem to be a more widespread phenomenon. As a consequence, a possible regulation of ribozyme activity by metal ions is an intriguing possibility [33]. Mg^{2+} and Ca^{2+} occur solvated in the cell plasma, but others like Mn^{2+} could be specifically delivered by proteins.

(iii) Metal ions other than Mg^{2+} often bind with considerably higher affinity than Mg^{2+} itself. In the case of transition or d^{10} elements, this is no surprise due to their relative positioning in the Irving-Williams series. This is not true for Ca^{2+} as for example its affinity to the RNA building block AMP^{2-} is 25% lower than that of Mg^{2+} [5,44]. Nevertheless, even at a ten-fold excess of Mg^{2+}, Ca^{2+} can completely inhibit splicing of a group II intron construct. Consequently, at least one Ca^{2+}-specific binding pocket must exist, where the affinity is more than 1'000x higher than for Mg^{2+} (to reach 99% inhibition). Such an increase in affinity only requires an additional binding energy of 17 $kJmol^{-1}$, i.e. corresponding to one additional hydrogen bond of a coordinated water molecule. Hence, together with the increased ionic radius (66 vs. 99 pm) and higher maximal coordination number (6 vs. 8), such specific binding pockets can be easily envisaged.

(iv) The observed folding heterogeneity of a group II intron ribozyme in the presence of Ca^{2+} by smFRET could also be true for other ribozymes when being accelerated with different metal ions. The small FRET differences observed with the group II intron make it impossible to see the same in bulk experiments. Consequently future smFRET studies might reveal highly interesting and unprecedented aspects of how different metal ions influence and control folding and activity of

ribozymes.

(v) Evaluation of the available data in the recent literature shows that in the HH ribozyme the rate is majorily governed by the metal ion affinity to the phosphate group(s) (Figure 4B) following a clear trend. It will be interesting to see if this is also true for other catalytic RNAs, if not for the more complex large ones, than at least for the small ribozymes.

Conflicts of interest

The authors declare no conflict of interest

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Figure Legends

Figure 1. Metal ion binding pocket in the major groove of the branch site in domain 6 of the Sc.ai5γ ribozyme [45,46]. A multitude of possible coordination sites for a metal ion (oxygens in red, nitrogens in blue) as well as one H-bond donor (NH_2 in green) are observed. It is highly plausible that depending on the size and coordinating properties of a given metal ion, a specific network of hydrogen bonds and coordination patterns for each distinct metal ion can be achieved in such a pocket. This Figure was prepared with MOLMOL (www.mol.biol.ethz.ch/groups/wuthrich_group/software) using PDB ID 2AHT [46].

Figure 2. (**A**) Overlay of crystal structures of HH ribozymes in the presence of Mg^{2+} (green spheres, PDB ID 301D) [36], Mn^{2+} (blue spheres, PDB ID 300D) [36], and Cd^{2+} (yellow spheres, PDB ID 488D) [37]. Mg^{2+} and Mn^{2+} occupy corresponding regions, but coordinate differently, whereas Cd^{2+} preferes other binding sites. (**B**) Close-up of the active site: The Mn^{2+} is bound inner-sphere both to the pro-R_P phosphate oxygen at nucleotide A9 and N7 of G10.1 (PDB ID 2OEU) [28]. Mg^{2+} also coordinates inner-sphere to the same phosphate oxygen but outer-sphere to the respective N7 position as indicated by the large distance of 4.5 Å (PDB ID 2QUW) [47]. The panels were prepared with MOLMOL (www.mol.biol.ethz.ch/groups/wuthrich_group/software).

Figure 3. (**A**) Metal ion coordinating atoms and pK_a values of RNA nucleobases. The pK_a values are taken from references [49], [42] and [50]. In the case of gua and ura the effect of a single-charged phosphate group was additionally taken into account. (**B**) The effect of N7 substituents on the pK_a of the guanine N1H position. The ΔpK_a values are taken from reference [40] to calculate the expected values p$K_{a,ex}$ values based on pK_a = 9.36 as given in (A).

Figure 4. Comparison of the observed rates of HH cleavage k_{obs} in the presence of various divalent metal ions with the affinities of these M^{2+} towards different ligands. (**A**) Classical Irving-Williams series as observed for metal ion binding to acetate (Δ), imidazole, and (□)NH_3 (◊) [48]. No overall correleation with the k_{obs} values from RzB HH (■) [15] and the *Schistosoma* HH (▲) [24] cleavage is observed. (**B**) The observed RzB HH (■) [15] and *Schistosoma* HH (▲) [24] cleavage rates k_{obs} correlate nicely with the affinities [5,42] of the respective M^{2+} towards a phosphate monoester with $pK^{H}_{H(R-MP)}$ = 6.20. This strongly suggests that the strength of the phosphate coordination dominates the metal ion effect in the HH ribozyme. Further properties like the pK_a of coordinated water molecules then determine, e.g. the acid-base profile of catalysis.

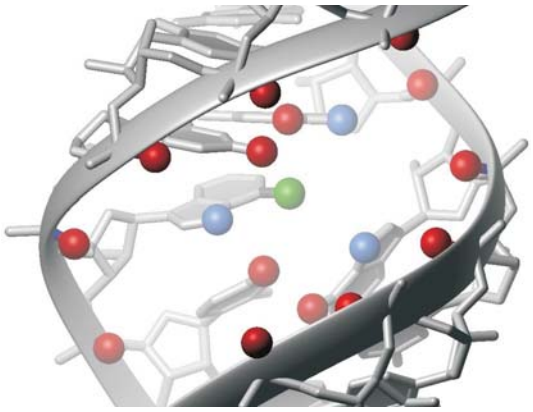

Figure 1

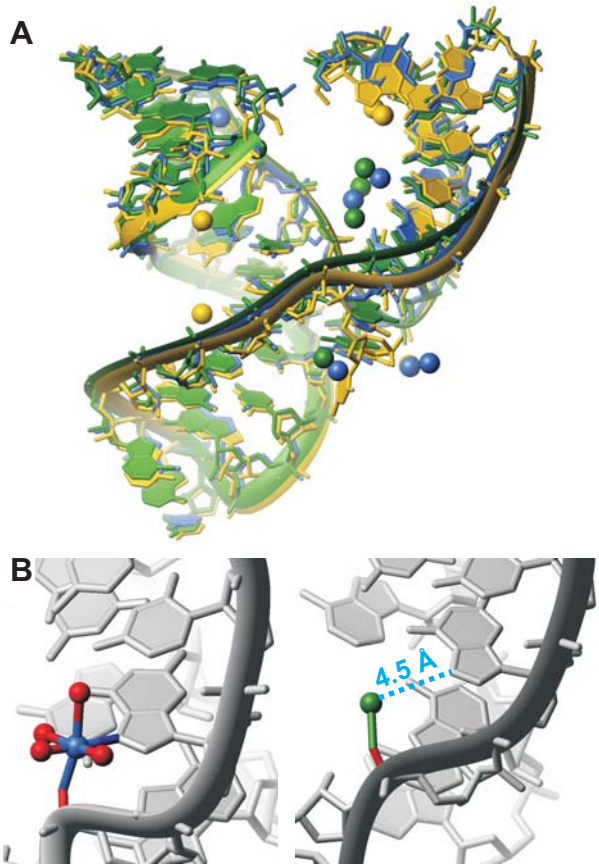

Figure 2

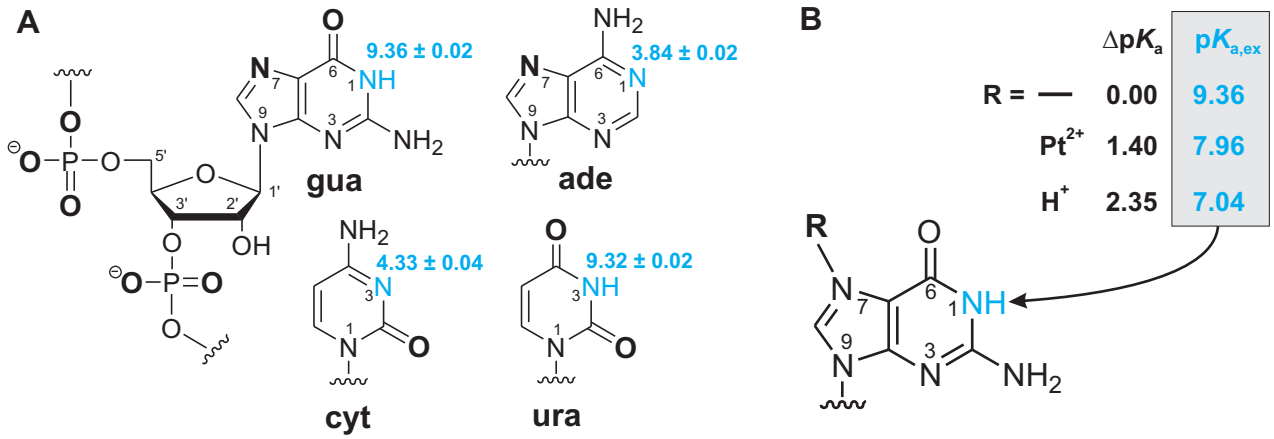
A
9.36 ± 0.02
gua
3.84 ± 0.02
ade
4.33 ± 0.04
cyt
9.32 ± 0.02
ura
B
ΔpKa
pKa,ex
R = —
0.00
9.36
Pt2+
1.40
7.96
H+
2.35
7.04
R

Figure 3

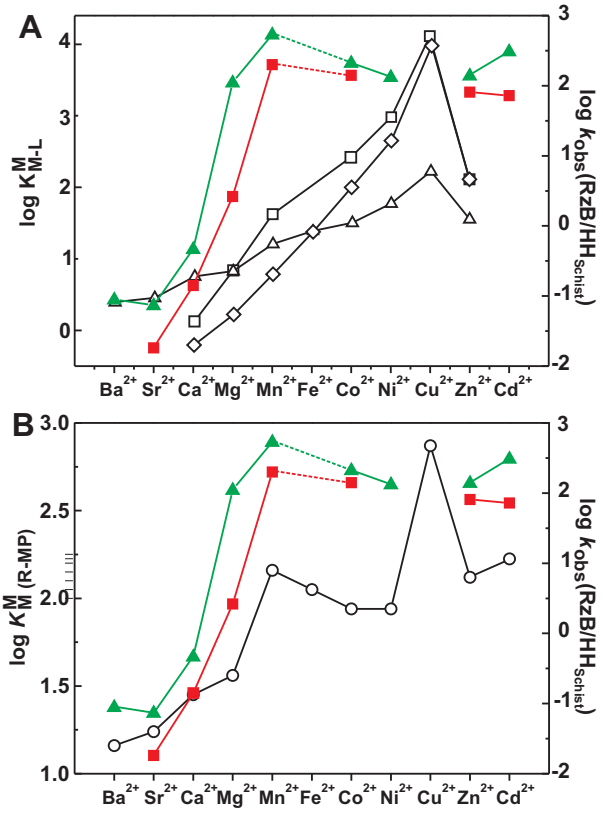
A
B
log K^{M}_{M-L}
log $K^{M}_{M(R-MP)}$
log k_{obs}(RzB/HH$_{Schist}$)
Ba^{2+} Sr^{2+} Ca^{2+} Mg^{2+} Mn^{2+} Fe^{2+} Co^{2+} Ni^{2+} Cu^{2+} Zn^{2+} Cd^{2+}

Figure 4